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FOREWORD

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INTRODUCTION

Estrogen and progesterone are critically important in the development and maintenance of reproductive tissues. These hormones exert their actions through intracellular receptors, which activate transcription by binding to hormone responsive elements (HREs, Reviewed in 1). The progesterone receptor (PR) is a transcription factor, which binds progestins and alters expression of progestin-responsive genes. The PR gene is responsible for the production of two distinct isoforms (2-6), the 120 kD PR-B, which contains a 164 amino acid amino-terminal region that is absent in the 94 kD PR-A isoform. PR-A and PR-B may arise through alternate initiation of translation (7) or transcription from alternate promoters present in the PR gene (8).

In normal mammary and uterine cells and in many human breast cancer cells, including MCF-7 cells (9), the PR gene is under estrogen control (8, 10-14). The increase in PR levels in breast cancer cells after exposure to estrogen has often been used as evidence of a functional estrogen receptor (ER). Whereas many early stage breast tumors maintain their responsiveness to estrogen, there is an unavoidable progression towards hormone insensitivity (9). Hormone-insensitive tumors typically have increased rates of proliferation and a loss of both ER and PR. Accordingly, screening of mammary tumors for PR has become an important clinical indicator of disease progression and hormone responsiveness. Understanding the mechanism by which this estrogen-responsive gene is regulated may help to define how mammary tumors progress from an estrogen-sensitive to an estrogen-insensitive state. In addition to regulation by estrogen, several studies have demonstrated that PR levels in MCF-7 cells are also modulated by antiestrogens, including tamoxifen, 4-hydroxytamoxifen and ICI 182,780 (13, 15, 16). The delineation of DNA sequences that are important in estrogen induction and antiestrogen silencing

of the PR gene in MCF-7 cells may help define how estrogens activate and antiestrogens suppress estrogen-responsive genes.

Although several models for steroid-induced gene transcription have been proposed, the exact mechanism by which steroid/nuclear receptors activate gene expression *in vivo* has yet to be defined. In order to address this issue, most studies have introduced chimeric plasmids into cultured cells. These studies, however, have several limitations. The DNA sequences introduced into cells are not endogenous sequences and are not phased into nucleosomes. Also receptors expressed by plasmid DNA are at superphysiological levels and may not be associated with appropriate accessory factors, which are present in limiting amounts.

With the advent of new technology, it is now possible to examine endogenous genes in their naturally occurring DNA topology using genomic footprinting coupled with ligation mediated polymerase chain reaction (LMPCR, Ref. 17, 18). This footprinting procedure, described by Mueller and Wold (17), demonstrated that the regulation of a single copy, inducible gene can be successfully examined in living cells. We have used this *in vivo* footprinting procedure to examine the 5' flanking region of the endogenous, estrogen-responsive PR gene. The region of the PR gene containing an ERE half site in close proximity to two Sp1 binding sites (+571 to +595) was examined to delineate protein-DNA interactions that occur in the absence and in the presence of estrogen. Using DNase I footprinting we find that regions flanking the ERE/Sp1 binding sites are minimally protected in the absence of hormone and more extensively protected in the presence of estrogen. Furthermore, dimethylsulfate (DMS) footprinting indicates that the Sp1 binding sites, but not the ERE half site, are protected in the presence and in the absence of estrogen. Taken together these results suggest that the Sp1

protein is bound to its binding sites before hormone treatment and that recruitment of transcription factors to regions flanking the ERE/Sp1 sites may help to mediate estrogen action.

BODY

Experimental Methods, Assumptions, and Procedures

Cell Culture MCF-7 cells (9), which had been maintained in Eagle's Minimum Essential Medium (MEM) containing 5% heat-inactivated calf serum, were seeded on 10 cm² plates and transferred to phenol red free, serum free media (19) five days before the experiments were conducted. The cells were exposed to ethanol vehicle, 1 nM 17 β -estradiol, 100 nM 4-hydroxytamoxifen or 100 nM ICI 182,780 for 2, 24, or 72 hours prior to DMS or DNase I treatment.

In vitro and in vivo treatment of genomic DNA Isolation of genomic DNA was carried out essentially as described by Mueller and Wold (18). Briefly, for DNase I footprinting, vehicle or hormone-treated MCF-7 cells were permeabilized with 0.4% NP-40 and treated with 750 U DNase I / ml for 3 min at 25°C. For DMS footprinting, vehicle or hormone-treated MCF-7 cells were treated with 0.1% DMS in media for 2 minutes at 37°C while still plated on tissue culture dishes in order to limit perturbation of the DNA structure. Cells were lysed by addition of buffer containing 300 mM NaCl, 50 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 0.05% SDS, and 0.2 mg/ml proteinase K for 5 minutes at room temperature. The resulting cell slurry was incubated 3-5 hours at 55°C to digest cellular proteins. The genomic DNA was extracted twice with buffer-saturated phenol, twice with phenol/chloroform/isoamyl alcohol, once with chloroform/isoamyl alcohol, precipitated with one volume of isopropanol, and transferred by spooling of the DNA onto a thin glass rod to 500 μ l TE, pH 7.5 (10 mM Tris pH 7.5, 1 mM

EDTA pH 8.0). The DNA was reprecipitated, resuspended in 100 μ l TE, incubated with 10 μ g of RNase A for 30 min at 37° C to digest cellular RNA, extracted twice with phenol/chloroform/isoamyl alcohol, reprecipitated with sodium acetate and ethanol, and resuspended in 200 to 500 μ l TE, pH 7.5. Methylated genomic DNA, which had been isolated from *in vivo* DMS-treated cells, was precipitated with ethanol and cleaved in 200 μ l of 1 M piperidine/175 μ g DNA for 30 minutes at 90°C, then chilled on dry ice for 10 minutes and evaporated in a Speedvac until dry. The pellet was resuspended in TE, pH 7.5 and precipitated with ethanol. The pellet was resuspended in 50 μ l water and evaporated to dryness to remove any remaining piperidine. The resuspension and evaporation procedures were repeated 2-6 times. Finally, the pellet was resuspended in TE so that the final DNA concentration was approximately 1 μ g/ μ l. The DNA solution was stored at -20 °C.

In vitro methylated DNA was prepared by adjusting 100 μ g of protein-free DNA to 175 μ l with TE, pH 7.5. Treatment of DNA with DMS was carried out as described (18). The reaction was stopped by the addition of sodium acetate and ethanol, then precipitated on dry ice. The methylated DNA pellet was resuspended in 200 μ l of 1 M piperidine and processed as described above. *In vitro* DNase I-treated DNA was prepared by adjusting 100 μ g of protein-free, RNase A-treated DNA to 175 μ l with TE, pH 7.5. DNA was incubated with 2.5×10^{-5} U DNase I for 5 min at 37° C. The reaction was stopped by the addition of 10 mM EDTA, pH 8.0, and processed as described for *in vivo*-treated genomic DNA.

In vivo footprinting LMPCR footprinting was carried out essentially as described by Mueller and Wold (17, 18). 1-2 μ g of genomic DNA was subjected to LMPCR procedures using nested primers, which annealed to sequences 5' of the ERE/Sp1 sites (+571 to +595) in the human PR

gene. The primers were: Primer 1- 5'TCCCCGAGTTAGGAGACGAGAT3', Primer 2- 5'CGCTCCCCACTTGCCGCTC3', and Primer 3- 5'GCTCCCCACTTGCCGCTCGCTG3'.

The annealing temperature for the primers were 55°, 62°, and 69°, respectively. The linker primers LMPCR 1 and LMPCR 2 described by Mueller and Wold (17) were also used, except that LMPCR 1 was modified by removing the two 5' nucleotides to eliminate potential secondary structure.

Results

Optimization of the LMPCR footprinting procedure

LMPCR footprinting requires the utmost attention to detail and careful analysis of each step involved. In order to successfully carry out the LMPCR footprinting procedure, a number of steps were optimized.

During the course of our investigations, we determined that the design of the primers used was critical for the success of the LMPCR footprinting procedure. To examine the ERE/Sp1 sites, we designed and purchased three sets of nested primers. The first set of primers were designed so that the end of Primer 3 was located approximately 25 bp from the ERE/Sp1 sites. These oligos tended to produce footprints that were somewhat obscured by the labeled primer. In addition, one of these primers may have annealed to other nonspecific sites in the genome because the footprints produced were not particularly clear. The second and third sets of primers were designed using the Oligo software program (National Biosciences Incorporated, Plymouth, MN) to try to limit primer-dimer pairing and nonspecific annealing of the oligos to other DNA regions. The second set of oligos were designed to anneal to the noncoding strand and have been used successfully to examine the ERE/Sp1 sites in the *in vivo* DMS and DNase I footprinting

experiments presented in this report. The third set of oligos were designed to anneal to the coding strand 50 bp from the ERE/Sp1 sites. Although this is somewhat closer to the region of interest than desired, the oligos were located at this position to try to avoid annealing to a potential hairpin structure 75 bp from the ERE/Sp1 sites. We were, however, unable to obtain footprints using these primers most likely due to secondary structure formation. However, we will continue to try to work out conditions to examine the coding strand so that we will have additional information about protein-DNA interactions in this region.

The annealing temperatures were optimized for all three nested primers for the first strand synthesis, amplification, and labeling reactions and are listed in the Methods section. In addition, the magnesium concentration was optimized to produce a clear footprint. We found that 4.5 mM MgCl_2 was optimal for all three steps with the primers used.

Initially we had problems with DNA purity and were unable to successfully carry out the LMPCR procedure. We have found that Proteinase K digestion of cellular proteins at 55° C for 4 hours rather than the 37° C recommended by the original procedure (17) and the use of high quality phenol were necessary. We have now optimized the DNA purification procedures sufficiently so that the results are much more reproducible.

DNase I and DMS concentrations were optimized so that cleavage was neither too limited nor too excessive. In addition, both whole cells and isolated nuclei were exposed to DNase I. Several DNase I concentrations were used with isolated nuclei (40-240U/ml) and intact, adherent cells (250-1500 U/ml). We found that 80 U/ml and 750 U/ml provided appropriate cleavage of the genomic DNA present in nuclei and whole cells, respectively. All of the data presented in this report are from experiments carried out with whole cells. Likewise, after testing several

concentrations and times of DMS exposure, we found that 0.1% DMS for 2 min at 37° C provided optimal cleavage of the genomic DNA. Conditions were also optimized so that the DNA exposed to DNase I or DMS *in vitro* was not excessively cleaved and so that the *in vitro* DNase I-cleaved DNA matched the *in vivo*-cleaved DNA. In addition, we have discovered that RNase I-treatment of DNase I-cleaved samples decreased background problems substantially.

We have optimized the labeling procedure by extending the kinasing reaction from 30 minutes to 1 hour and using new kinase and γ [³²P]ATP. We can now more reliably label Primer 3 to a specific activity of greater than 20×10^6 cpm/pmol DNA.

DNase I footprinting reveals regions of protection flanking the ERE/Sp1 binding sites.

To help define how estrogen is involved in the regulation of the endogenous PR gene, we used *in vivo* DNase I footprinting to examine the PR 5' flanking region in its normal DNA context in living MCF-7 cells. One region of particular interest in our investigation of the PR gene were nucleotides from +571 to +595, a region which contains an ERE half site located in close proximity to two Sp1 binding sites (Fig. 1, Ref. 8). Using LMPCR footprinting, we examined this region to identify DNA regions that were protected by proteins. MCF-7 cells were treated with ethanol vehicle or 17 β -estradiol for 2 hours or 72 hours before exposure to the NP-40/DNase I mixture. The cells were harvested, DNA was isolated, and LMPCR procedures were carried out. Naked genomic DNA was subjected to *in vitro* DNase I cleavage to serve as a reference in identifying sequences susceptible to cleavage in the absence of proteins.

Comparison of *in vitro* and *in vivo* DNase I-treated samples indicated that very few nucleotides flanking the ERE/Sp1 binding sites were protected when MCF-7 cells were maintained in a hormone-free environment (Fig. 2, Compare V_i and 0 h E₂). However, when MCF-7 cells were

treated with E_2 for 2 hours, regions 5' and 3' of the ERE/Sp1 sites were much more extensively protected (Fig. 2, 2h E_2). When MCF-7 cells were treated with E_2 for 72 hours (Fig. 2, 72h E_2), a time period when PR mRNA and proteins reach maximal levels (2-6), the protection of regions 5' and 3' of the ERE/Sp1 sites was more extensive than at 2 hours of estrogen treatment. Thus, we were able to define differences in protection of the regions outside of the ERE/Sp1 sites after estrogen treatment using DNase I footprinting. We were, however, unable to define in any detail the interaction of proteins with the ERE/Sp1 sites. Multiple attempts to examine this region demonstrated that neither naked genomic *in vitro* DNase I-treated nor *in vivo* DNase I-treated genomic DNA was susceptible to cleavage in the ERE/Sp1 sites.

DMS footprinting delineates guanine residues involved in modulating PR expression

We next utilized DMS *in vivo* footprinting to determine if we could obtain more detailed information about the protein-DNA interactions occurring within the ERE/Sp1 sites. This footprinting method utilizes DMS, a small molecule, to modify guanine residues and can identify specific guanine residues involved in interacting with proteins. The modified guanine residues are cleaved with piperidine after purification of the genomic DNA.

MCF-7 cells were treated with ethanol vehicle or hormone for 24 hours and then exposed to DMS in order to methylate guanine residues that were not intimately associated with proteins. Cells were lysed, DNA was isolated, methylated guanine residues were cleaved with piperidine, and LMPCR procedures were carried out. When MCF-7 cells were maintained in a hormone free environment, guanine residues in both Sp1 binding sites were protected. Upon exposure of cells to E_2 , two additional guanine residues in one of the Sp1 sites were protected and residues flanking the ERE half site and adjacent Sp1 site displayed enhanced sensitivity to DMS (Fig. 3,

E₂). Interestingly, none of the guanine residues in the ERE half site appeared to be protected in the presence or in the absence of estrogen treatment.

Treatment of MCF-7 cells with antiestrogens elicits unique footprinting patterns

A subject of great clinical interest has been to delineate how tamoxifen, an antiestrogen with agonistic and antagonistic properties, and ICI 182,780, a pure antiestrogen, inhibit disease recurrence in breast cancer patients (20-22). Although the effects of these compounds have been studied using *in vitro* DNA binding assays and transient transfection assays, their effects on protein-DNA interactions in a native gene have not been addressed.

When MCF-7 cells were treated with 4-hydroxytamoxifen, the footprinting pattern observed was quite similar to that of *in vitro* DNase I-treated naked DNA except that one region just outside of the Sp1 site displayed enhanced sensitivity to DNase I (Fig. 4, T). This enhanced hypersensitivity can result from protein-induced conformational changes brought about by protein binding (23). Thus, treatment of MCF-7 cells with 4-hydroxytamoxifen resulted in minimal changes in the protection of this region of the PR 5' flanking region. When MCF-7 cells were treated with ICI 182,780, a very different and distinct footprinting pattern was observed. Several residues adjacent to the Sp1 sites were protected and the region of hypersensitivity was diminished (Fig. 4, I). Thus, the two antiestrogens tested, one a partial agonist/antagonist and the other a pure antagonist, produced very different footprinting patterns.

Discussion

The most commonly used method to define how hormones regulate transcription of target genes has been to use transient transfection assays. Using this technique, Kastner et al. (8) hypothesized that the human PR gene may contain two separate promoters, which are responsible

for the production of A and B receptors. Both the B (-711 to +31) and the A (+464 to +1105) promoters were shown to be estrogen responsive when fused to a CAT reporter plasmid and transfected into HeLa cells (8).

Since EREs are involved in conferring estrogen-responsiveness to many genes, a number of investigators have been sought to identify ERE sequences in the PR gene. The rabbit PR gene contains an imperfect ERE that overlaps with the translation start site and is capable of conferring estrogen responsiveness to a heterologous promoter in transfection assays (24). Although a similar sequence has been identified in the chicken PR gene (25), no homologous sequence is present in the human PR gene (8). However, an ERE half site was identified at +571 in the human PR gene (Fig. 2B), just upstream of two putative Sp1 binding sites (8). Sp1 binding sites have been implicated in the estrogen responsiveness of the creatine kinase B (26), c-myc (27), retinoic acid receptor α (28), heat shock protein 27 (29, 30), and cathepsin D (31) genes. Moreover, like the PR gene, all five of these promoters have an ERE half site located in close proximity to an Sp1 binding site (26-31). It has been hypothesized that the Sp1 protein and the ER may act cooperatively to mediate the estrogen-induced transcription of these genes. Antibody supershifts carried out with ER and Sp1 demonstrated that these proteins bind as a complex to a Sp1/ERE oligonucleotide (29, 30). Although deletion or mutation of either the Sp1 or the ER binding sites was shown to decrease estrogen-induction and the formation of a gel shifted band in early studies (29), more recent investigations have demonstrated that the Sp1 binding site, but not the ERE half site was necessary for Sp1 binding (30). Porter et al (30) suggest that the ER may promote binding of the Sp1 protein to its binding site and may be associated with the Sp1 protein, but that the ER is not associated with the ERE half site.

We have examined the region of the PR gene containing an ERE half site in close proximity to two Sp1 binding sites (+571 to +595) in order to delineate protein-DNA interactions that are important in conferring the effects of estrogen and antiestrogens. Unlike previous studies, which used supercoiled plasmids in transient transfection assays of isolated promoter regions, we have utilized *in vivo* genomic footprinting coupled with PCR amplification to identify cis elements responsible for hormonal regulation of the endogenous PR gene in its natural DNA topology. DMS and DNase I footprinting provides complementary information about the ERE/Sp1 sites and adjacent sequences involved in PR regulation. We find that estrogen exposure elicited more extensive protection of the Sp1 sites and DNA sequences in the region of the ERE/Sp1 binding sites than was observed in the absence of hormone. Interestingly, the ERE half site was not occupied in the absence or in the presence of hormone, indicating that the ER is most likely not bound to this sequence. These findings provide evidence that the Sp1 sites, but not the ERE half site are involved in mediating estrogen-induced PR transcription and are consistent with the more recent *in vitro* binding assays of Safe and coworkers (30).

Our *in vivo* footprinting experiments demonstrated that the timecourse of PR mRNA production parallels the events occurring at the level of the gene. Estrogen treatment of MCF-7 cells results in maximal PR mRNA levels after 3 days (8, 10-14). Likewise, protein-DNA interactions in the region of the ERE/Sp1 sites increased and became more extensive after 2 and 72 hours of E₂ treatment (Figs. 2 and 3).

The effects of 4-hydroxytamoxifen and ICI 182,780 on protein-DNA interactions in the ERE/Sp1 region of the PR gene were quite distinct. The partial antagonist, 4-hydroxytamoxifen,

elicited a footprint that was very similar to naked genomic DNA which had been treated with DNase I *in vitro*, while the pure antagonist, ICI 182,780, produced a different, distinct footprint (Fig. 4). These findings are very similar to our studies of the estrogen-responsive pS2 gene, in which 4-hydroxytamoxifen elicited a footprint that was very similar to that of the control-treated MCF-7 cells (32). Thus, two estrogen-responsive genes with very different nucleotide sequence and cis elements respond to these two antiestrogens in a similar fashion.

The more extensive pattern of protection flanking the ERE/Sp1 sites suggests that this region is associated with a number of proteins. Numerous groups have reported the association of steroid hormone receptors with coactivators and corepressors (Reviewed in 33) and references therein). Recent studies have also identified coactivator and corepressor proteins with histone acetylase and deacetylase activities, respectively (34-37). Association of ER with these coregulators may be important in modulating chromatin structure and the accessibility of transcription factor binding sites in native chromatin. The divergent footprinting patterns observed with estrogen- and antiestrogen-treated MCF-7 cells suggest that unoccupied, estrogen-occupied, and antiestrogen-occupied ER associate with different sets of coactivator and/or corepressor proteins and that these proteins may in turn form an interconnected protein-DNA complex. Estrogen treatment could release corepressor proteins and promote interaction of the receptor with coactivators resulting in changes in local chromatin structure. Although future characterization of the protein-DNA complexes involved in regulation of the PR gene will be required, these *in vivo* footprinting experiments provide us with a more physiologically relevant view of how estrogens and antiestrogens bring about their effects in target cells.

Statement of Work

In this first year of the grant, we have addressed Specific Aims 1a, 1c, and 2a. The experiments carried out with antiestrogens (Specific Aim 2a) were altered slightly. Our original proposal, which included an examination of the effects of 4-hydroxytamoxifen on protein-DNA interactions, was extended to examine the effects of two different antiestrogens, 4-hydroxytamoxifen and ICI 182,780, on protein-DNA interactions in the region of the ERE/Sp1 sites. Instead of examining the effects of concurrent estrogen and tamoxifen treatment, decided it might be more clear-cut to investigate the effects of each antiestrogen alone. Also, we have included both DMS and DNase I *in vivo* footprinting to obtain additional information about the protein-DNA interactions within and surrounding the ERE/Sp1 sites rather than relying on DMS footprinting alone as originally proposed.

Specific Aim 1, Determine the effects of estrogen treatment on protein-DNA interactions in MCF-7 cells, Months 1-12

- a. Examine the ERE half site and Sp1 sites in promoter A for evidence of protein-DNA interactions after treatment of MCF-7 cells with ethanol vehicle or estrogen.
- b. Examine the CAAT motif and Sp1 sites in promoter B for evidence of protein-DNA interactions after treatment of MCF-7 cells with ethanol vehicle or estrogen.
- c. Determine how the length of estrogen treatment affects protein-DNA interactions.

Specific Aim 2, Determine the effects of concurrent estrogen and antiestrogen treatment on protein-DNA interactions in MCF-7 cells, Months 12-24

- a. Examine the ERE half site and Sp1 sites in promoter A for evidence of protein-DNA interactions after concurrent treatment of MCF-7 cells with estrogen and either 4-

hydroxytamoxifen.

- b. Examine the CAAT motif and Sp1 sites in promoter B for evidence of protein-DNA interactions after concurrent treatment of MCF-7 cells with estrogen and either 4-hydroxytamoxifen.

Figure Legends

Figure 1. Sequence of the human progesterone receptor gene. The sequence of the progesterone receptor gene from - 90 to +1336 (8) is shown.

Figure 2. *In vivo* footprinting of the progesterone receptor gene after exposure of intact MCF-7 cells to DNase I. MCF-7 cells were maintained in serum-free medium for five days, treated with ethanol control (0 h E₂) or 1 nM E₂ (E₂) for 2 or 72 hours, and then exposed to NP40/DNase I. Genomic DNA was isolated and used in *in vivo* LMPCR footprinting. Naked genomic DNA samples, which had been treated *in vitro* with either DNase I (V) or DMS (G), were included as references. The location of the ERE half site and the Sp1 sites are indicated. Nucleotides protected in control- or E₂-treated cells are indicated to the right of the autoradiogram. Solid bars indicate regions of DNase I hypersensitivity.

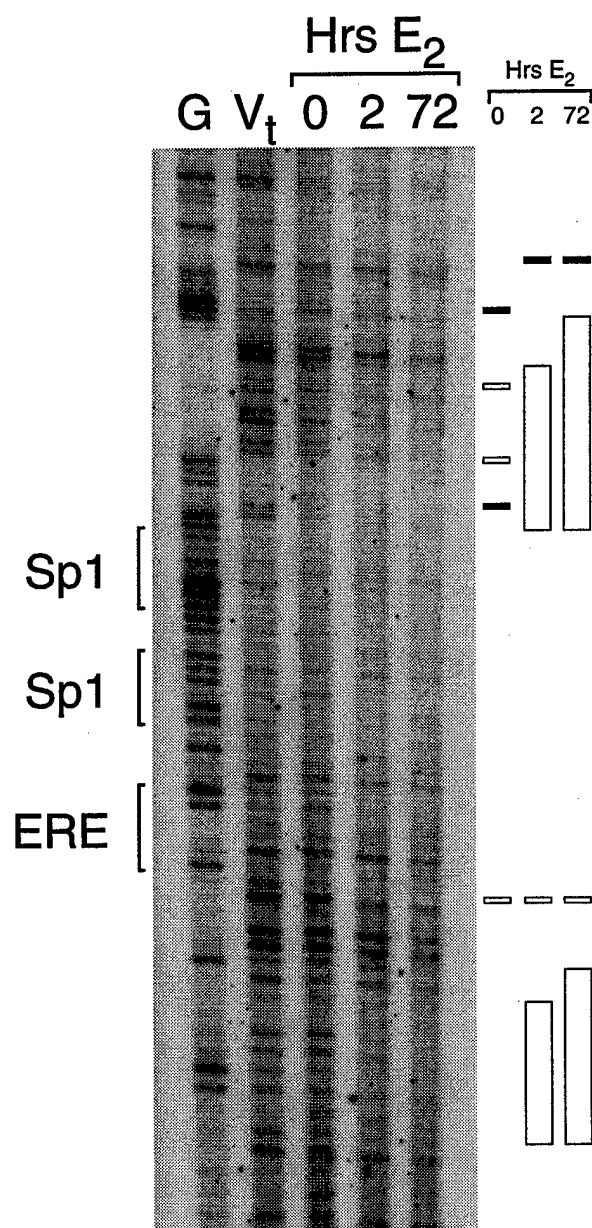
Fig. 3. *In vivo* footprinting of the progesterone receptor gene after exposure of intact MCF-7 cells to DMS. MCF-7 cells were maintained in serum-free medium for five days and then treated with either ethanol control (-) or 1 nM E₂ (E₂) for 24 hours. Genomic DNA was isolated and used in *in vivo* LMPCR footprinting. A naked genomic DNA sample, which had been treated *in vitro* with DMS (G), was included for reference. The location of the ERE half site and the Sp1 sites are indicated. Nucleotides protected in control- or E₂-treated cells are indicated to the right of the autoradiogram (open bars). Solid bars indicate regions of enhanced cleavage.

Fig. 4. *In vivo* footprinting of the progesterone receptor gene after exposure of intact MCF-7 cells to estrogen or antiestrogen and DNase I. MCF-7 cells were maintained in serum-free medium for five days and then treated with either ethanol control (-), 1 nM E₂ (E₂), 100 nM 4-hydroxytamoxifen (T), or 100 nM ICI 182,780 (I). Genomic DNA was isolated and used in *in vivo* LMPCR footprinting. The location of the ERE half site and the Sp1 sites are indicated. Naked genomic DNA samples, which had been treated *in vitro* with either DNase I (V_I) or DMS (G), were included as references. Nucleotides protected in 4-hydroxytamoxifen- or ICI 182-780-treated cells are indicated to the right of the autoradiogram. Solid bars indicate regions of enhanced cleavage.

Progesterone Receptor gene sequence

-90 agtttcagatcctaccggtaatggggtagggaggggcttggcggggcctccctagaggaggaggcggtttagaaagctgt
 +1
 -6 ctggccagtcacagctgtcactaatcgggtaagccttgttatttgcgcgtgtgggtggcattctcaatgagaactagcttcac
 82 ttgtcatttgagtgaaatctacaacccgagggcggtagtgtcccgactactgggatctgagatcttcggagatgactgtcgccc
 168 gcagtacggagccagcagaagtccgaccttctgggaatgggctgtaccgagaggtccgactagccccagggtttagtgag
 251 ggggcagtggaaactcagcaggggactgagagcttcacgcatgcagagtttgatgccagagaaaaagtcgggagataaagg
 333 agccgcgtgtcactaaattgccgtcgcagccgcagccactcaagtgccggacttgtgagtactctgcgtctccagtcctcggaca
 418 gaagtggagaactctcttgagaactccccgagtaggagacgagatctcctaacaattactacttttctgcgtccccacttgc
 half ERE Sp1
 506 cgctcgtgggacaaacgacagccacagtccccctgacgacaggatggaggccaagggcaggagctgaccagcgccccct
 Sp1
 587 ccccgccccgaccagggaggtggagatcctccggtccagccacattcaaccccactttctcctccctctgcccctatatccccc
 G/C rich B Receptor
 673 gaaacccccctcctccttccctttccctcctcctggagacgggggaggagaaaaggggagtcagtcATGACTGA
 Sp1 Sp1
 752 GCTGAAGGCAAAGGGTCCCCGGGCTCCCCACGTGGCGGGCGGCCGCCCTCC
 804 CCCGAGGTCGGATCCCCACTGCTGTGTGCGCCAGCCGCAGGTCCGTTCCCGGG
 857 GAGCCAGACCTCGGACACCTTGCCTGAAGTTTCGGCCATACCTATCTCCCTGGA
 911 CGGGCTACTCTTCCCTCGGCCCTGCCAGGGACAGGACCCCTCCGACGAAAAGA
 964 CGCAGGACCAGCAGTCGCTGTGCGGACGTGGAGGGCGCATATTCCAGAGCTGAA
 1017 GCTACAAGGGGTGCTGGAGGCAGCAGTTCTAGTCCCCCAGAAAAGGACAGCGG
 1070 ACTGCTGGACAGTGTCTTGGACACTCTGTTGGCGCCCTCAGGTCCCGGGCAGA
 1123 GCCAACCCAGCCCTCCCGCCTGCGAGGTACACAGCTCTTGGTGCCTGTTTGGCC
 1177 CCGAATTCCCGAAGATCCACCGGCTGCCCCCGCCACCCAGCGGGTGTGTGCCC
 A Receptor
 1231 CGCTCATGAGCCGGTCCGGGTGCAAGGTTGGAGACAGCTCCGGGACGGCAGCT
 1284 GCCCATAAAGTGCTGCCCCGGGGCCTGTCACCAGCCCGGCAGCTGCTGCTCCC

Figure 1

**Figure 2**

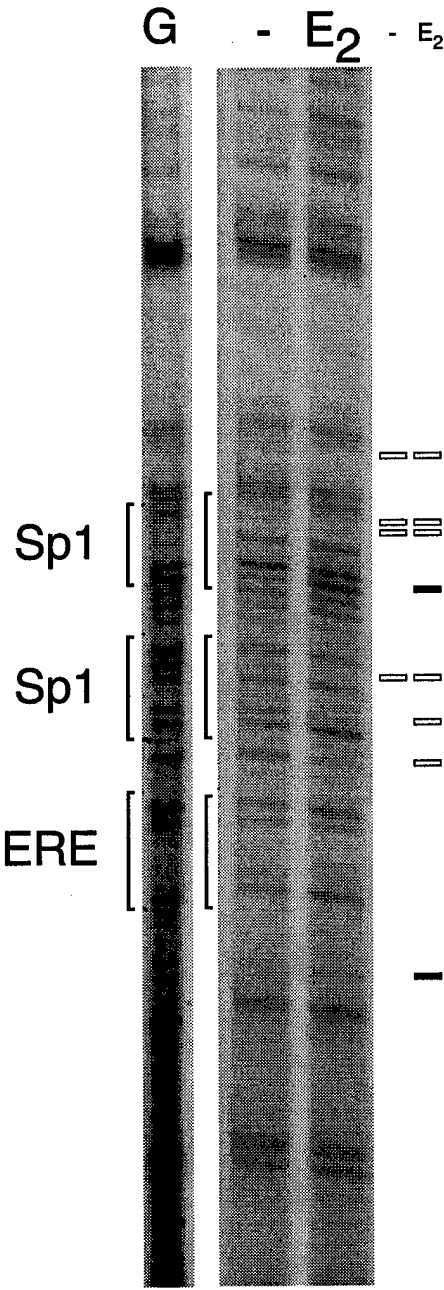
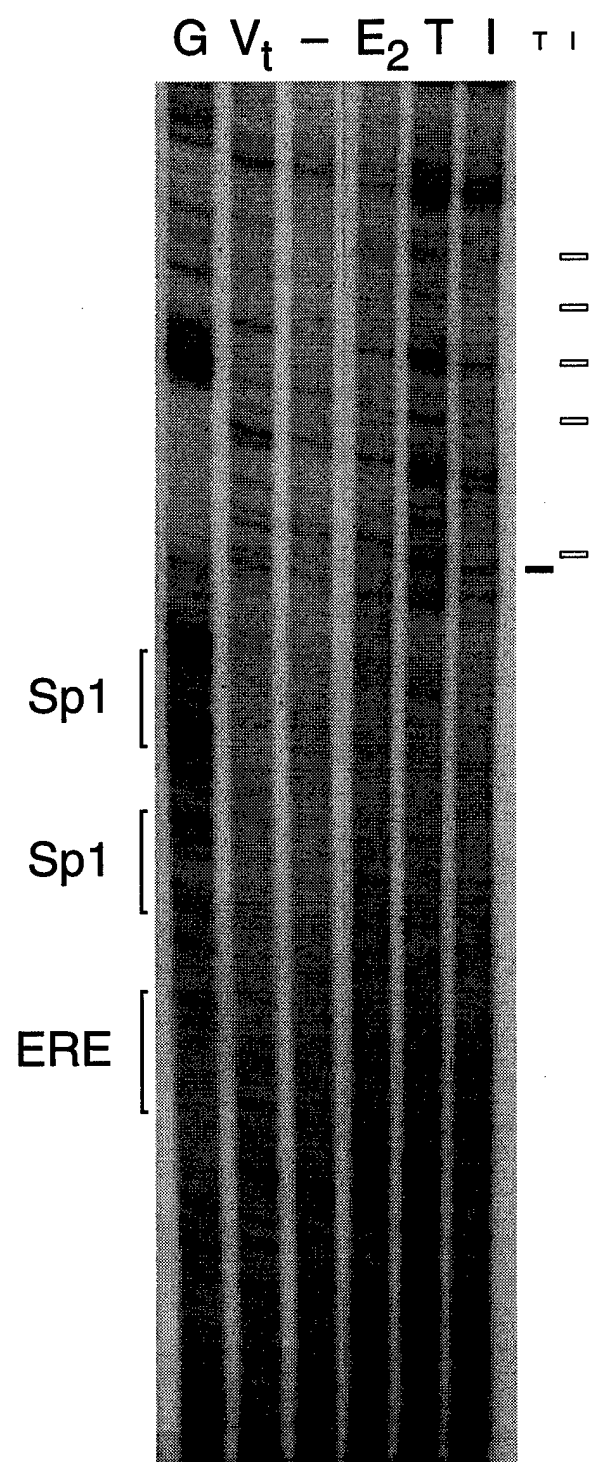


Figure 3

**Figure 4**

CONCLUSIONS

1. The conditions required to carry out *in vivo* DMS and DNase I footprinting of the PR gene have been defined.
2. DMS footprinting of the PR gene demonstrates that the Sp1 sites in the A promoter are protected in the absence of hormone, but more extensively protected in the presence of E₂.
3. DMS footprinting demonstrates that the ERE half site present in the A promoter is not protected in the presence or in the absence of E₂.
4. DNase I footprinting demonstrates that regions flanking the ERE/Sp1 sites are more extensively protected after E₂ treatment.
5. Preliminary DNase I footprinting experiments demonstrate that two antiestrogens elicited different footprinting patterns. The partial agonist 4-hydroxytamoxifen produced a footprinting pattern that was similar to that of *in vitro*-cleaved, naked genomic DNA. In contrast, the pure antagonist ICI 182,780 produced a footprinting pattern that was distinct from all other footprints observed.

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